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Immunofluorescent Detection of Staphylococcal Enterotoxin B in Food and Culture Media

Raymond Lloyd Stark

IMMUNOFLUORESCENT DETECTION OF STAPHYLOCOCCAL ENTEROTOXIN B IN FOOD AND CULTURE MEDIA

BY

RAYMOND LLOYD STARK

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This thesis is approved as a creditable and independent investigation by a candidate for the degree, Master of Science, and is acceptable as meeting the thesis requirements for this degree, but without implying that the conclusions reached by the candidate are necessarily the conclusions of the major department.

Thesis Advisor // Date

Head, Department of Bacteriology Date

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INTRODUCTION

The most commonly occurring true food poisoning is caused by the ingestion of enterotoxin formed in foods during the growth of certain strains of Staphylococcus aureus. The toxin is termed an enterotoxin because it causes gastroenteritis or inflammation of the lining of the stomach and intestine (22).

The number of staphylococci is not necessarily a reliable measure of the toxicity of a food incriminated in an outbreak. The heat stable enterotoxin may survive, whereas the organisms are destroyed in a heat processed food. The demonstration of enterotoxin in an implicated food is the only direct indication that the poisoning agent is staphylococcal enterotoxin. Four enterotoxins (A, B, C, D) have been serologically demonstrated.

Although many methods have been developed or investigated for the detection, identification, and quantitation of staphylococcal enterotoxin, the need still exists for a rapid method for its detection in foods. After the antigenic nature of enterotoxin was shown, gel-diffusion techniques were developed for the detection, identification, and quantitation of enterotoxin in foods. The gel-diffusion tests are reliable and sensitive but are time consuming and require extraction and concentration of enterotoxin from the food. Several methods have been tried for the detection of enterotoxin and all have shown some disadvantage. The fluorescent antibody technique has been shown to have much promise as a diagnostic tool in the detection of enterotoxin in culture media and foods.

Several workers have investigated the effect of some nutritional and environmental conditions on enterotoxin production, using gel-diffusion methods for the detection and quantitation of the enterotoxin. Most of these conditions have been investigated using culture media but not foods for the growth of Staphylococcus aureus. The effects of these conditions in foods and in competition with other organisms must be subject to further study so that standards and quality control measures may be established for foods.

This study was undertaken to investigate the possibility of using the fluorescent antibody technique to rapidly demonstrate and estimate enterotoxin concentrations in food and culture media. The correlation between degree of cell fluorescence and enterotoxin production of organisms grown under varying environmental and nutritional conditions was shown.

LITERATURE REVIEW

Food poisoning caused by staphylococcal enterotoxin is the most prevalent foodborne disease. The increased number of food poisoning outbreaks has brought about the need for improved techniques of controlling the disease (38).

Some strains of Staphylococcus aureus, usually coagulase positive, produce enterotoxins, substances capable of irritating the gastrointestinal tract (59). Enterotoxins are exotoxins formed at the surface of the cells. Enterotoxins are elaborated by growth of staphylococci in culture media and most importantly in certain food products. The food poisoning caused by staphylococcal enterotoxin is severe but usually non-lethal (20,27,54).

Unlike botulism toxin, which is destroyed rapidly by temperatures below boiling, crude enterotoxins are stable to boiling and even autoclaving (19,20,47). Enterotoxins are simple proteins having no interfering lipid or carbohydrate portions, which has allowed their purification and characterization (1,2,23). The demonstration of a specific precipitating antibody (3) has allowed the use of certain antigen-antibody reactions for the detection and characterization of enterotoxins (10). At least four antigenically distinct types of enterotoxin are known. Their classification (A,B,C,C_2,D) is based on their reactions with specific antibodies (9). Enterotoxin A causes most outbreaks of food poisoning, the prototype strain being S. aureus 196E. Although enterotoxin A is involved in most food poisonings, enterotoxin B is investigated more because it is easily purified and

is produced in large amounts by enterotoxigenic strains in culture media. Strain 243 is the prototype strain for enterotoxin B while strain S-6 produces both enterotoxins A and B. Types C and D have been defined only recently and their relative importance has not been established.

Hodge (38) has reported that 99% of the staphylococcal outbreaks that have been investigated were caused by enterotoxin formed in cooked high protein food. High levels of sugar and salt in foods inhibit many organisms, but not the growth of staphylococci. In the United States foods incriminated in outbreaks of staphylococcal intoxication include ham and other meat products, poultry and poultry dressing, sauces and gravies, potato salad, custard filled-pastries, bread pudding, cheese, and milk (6,19,22). Because there is a large variety of foods incriminated, a rapid detection technique for enterotoxin is needed.

The essential requirement to incriminate a food is the demonstration of enterotoxin in the food. Cats, kittens, frogs, and monkeys have been used in the assay of staphylococcal enterotoxin, but the results are not always reliable or consistent.

The purification (2,64) and the antigenic nature of enterotoxins have allowed the use of serological techniques. The production of antiserum (10) has brought about the use of various agar gel-diffusion techniques for the detection of enterotoxin. These techniques have been used with culture filtrates and concentrated food extracts, with a minimum sensitivity of 0.5 ug/ml (37). Two principles have been employed in removing and concentrating enterotoxin from food in order that it may be detected by serological techniques (7). One depends on

the selective adsorption of the enterotoxin from an extract of the food on an ion exchange column (11,15,36) and the other depends on the use of physical and chemical procedures for selective removal of food constituents from the extracts, leaving the enterotoxin in solution. The extract must then be concentrated (55).

Of the serological procedures available for the study of enterotoxins, precipitation in gel has proved to be the most useful. It provides the simplest procedure for attaining the optimal proportions of antigen and antibody necessary for precipitation. The single diffusion test as described and modified by Hall and Weirether (36,66), the double diffusion test of Oakley and Fulthorpe (52), and the slide double diffusion technique as described by Crowle and Wadsworth (18,65) have been the most widely used techniques for the assay of enterotoxin (11). All of these methods require from 1 to 7 days before lines of precipitation are obtained. The single gel-diffusion test has been modified by Weirether (66) so that quantitative results are obtained within 4 to 16 hr with μg/ml of enterotoxin B.

Hopper (39) devised a flotation antigen-antibody system using rhodamine (fluorescent dye) labeled antiserum which was added to a toxin solution. The antigen-antibody complex was recovered in a colored dye-toxin complex in the foam produced by the system. The method is very rapid (1 min) but it lacks quantitative potential and requires more toxin than is usually found in incriminated foods.

Passive and reversed hemagglutination for the detection of enterotoxin have been investigated (41,60) and have been shown to be

very rapid and sensitive. Results were variable because of hemagglutinins produced by some staphylococci which are difficult to absorb or to destroy by heat.

The microtiter hemagglutination-inhibition method has been used for the assay of enterotoxin B from culture filtrates and pure enterotoxin (49). With this technique many samples can be assayed simultaneously and reliable results can be obtained in 3 hr. This method has been shown to be fast, requires only small quantities of reagents, is easily read and has good reproducibility. However, the hemagglutination assays have been obtained only with culture filtrates and not with foods or food extracts.

Fluorescent Antibody Technique (FAT)

A valuable serological tool for the rapid identification of microorganisms and their antigens became available with the introduction of the Coons fluorescent antibody technique (FAT) (15,16). The FAT is essentially an immunochemical staining procedure which permits microscopic observation of an antigen-antibody reaction. A protein or antibody is labeled with a fluorochrome (usually fluoresein isothiocyanate) according to the following chemical reaction (4).

The fluorochrome-antibody complex is termed a labeled antibody (FA) or conjugate. The conjugation of the labeled antibody and its homologous

antigen results in a product that fluoresces when viewed with the fluorescence microscope.

The simplest FAT procedure, the direct method, involves the direct application of the conjugate on smears of cultures. The direct method can also be used by mixing the FA with the antigen in solution. The stained preparation is then observed for stained organisms or fluorescent precipitates (12,34).

Improvements in the fluorescent antibody technique have allowed many new applications in diagnostic bacteriology. This has been brought about by the development of pure fluorochromes and improved techniques of preparing and using the labeled antibody (21,42,43,44,51, 53). The speed and sensitivity of the FAT has allowed for its wide use in bacteriology.

FAT For Detection of Enterotoxin

Because staphylococcal enterotoxins are antigenic and stimulate antibody production, workers have investigated the possibility of using the FAT for direct detection of enterotoxin B and enterotoxin producing cells in culture media and foods using conjugated antienterotoxin sera. The FAT was first used to demonstrate coagulase positive strains of S. aureus (5). Friedman and White (26) reported the demonstration of cell-associated staphylococcal enterotoxin B by immunofluorescence. This work was not complete and offered few details of techniques nor did they discuss the potential of the FAT for the detection of enterotoxin. Genigeorgis and Sadler (17,29,30) have shown that the FAT can be used to detect enterotoxin and enterotoxin producing cells in culture media

methods. The FAT has the potential to demonstrate directly cell-associated enterotoxin.

The investigator's purpose was to determine the correlation between enterotoxin production and cell fluorescence under varying conditions in food and culture media using two strains of S. aureus. The organisms were grown under certain nutritional and environmental factors such as NaCl concentration, temperature, initial pH, glucose concentration, and atmosphere which were varied and correlations were established from these data.

Shrimp was chosen as the main food for this study because it is high in protein and has been included in many studies on frozen foods (58,61,62). Beef pie and frozen peas were used to determine whether there is any interference in different protein foods.

MATERIALS AND METHODS

Sources of Staphylococcus Strains

Enterotoxigenic strains of Staphylococcus aureus were obtained from three sources, Dr. C. Genigeorgis, Department of Public Health, University of California, Davis; Dr. E. P. Casman, Food and Drug Administration, Washington D. C.; and Dr. R. H. Jones, School of Public Health, University of Michigan, Ann Arbor.

The following two groups of Staphylococcus strains were used in this study:

Enterotoxin Type	Enterotoxigenic Strains
A	196E, 100, 238, 239, 265-1
B	243 (ATCC 14458)
A and B	S-6
C	361, 137

Nontoxigenic strains

Coagulase positive: Wood 46
Coagulase negative: Staphylococcus albus

Strains 243 (the prototype for enterotoxin B) and S-6 were used throughout the study. The effect of nutritional and environmental conditions on cell fluorescence and enterotoxin B production was determined by using these two strains.

Staphylococcus aureus strains capable of producing enterotoxins A and C, Streptococcus faecalis and S. albus were used as negative

was given intravenously. At nine weeks the animals were bled from the marginal ear vein and injected again with 1000 µg. At fourteen weeks a cardiac bleeding was performed. The antiserum was checked for activity against enterotoxin B using the double gel-diffusion technique. The titer was found to be 1:80. Merthiolate (Eli Lilly and Co.) 1:10,000 was added to all sera, and the sera were stored at -20 C.

Oakley-Fulthorpe (Double gel-diffusion) (37,52)

The double gel-diffusion technique was used to determine the titer of the antiserum produced and to assay for enterotoxin B in food and culture media to verify results obtained with the fluorescent antibody technique.

Small test tubes (4 mm x 50 mm) were cleaned and placed in plastic test tube racks. The buffer was prepared as follows:

Merthiolate	1:10,000
Sodium barbital	0.80 g
Sodium chloride	0.85 g
Distilled H_2O	100 ml

Adjusted to pH 7.4

Six grams of Noble agar were added to the buffer, and the buffer was brought to a boil and then cooled to 50 C and held at this temperature. The desired dilution of antiserum was prepared and 1.5 ml was added to a small vial, to which 1.5 ml of agar solution was added. After thorough mixing, the mixture was then added to tubes in a rack (0.2 ml per tube), and allowed to solidify. After the antiserum-agar mixture had solidified, 0.2 ml of agar was added to each tube and also allowed to solidify. The material to be assayed was added to fill the

tube. Tubes were sealed with a square of parafilm and incubated at room temperature for 3 to 5 days and observed daily for lines of precipitation (36).

Slide Gel-Diffusion Test

The slight modification of the slide gel-diffusion test, as described in detail by Crowle (18), was using the same buffer as used for the Oakley-Fulthorpe technique. The use of plastic templates was also modified, in which the distance between the centers of the central and peripheral well is 4.5 mm instead of 4.0 mm, as described by Hall et al.(36). A detailed description for preparing and cleaning slides was obtained from Dr. E. P. Casman. After preparation, the slides were incubated for 3 days at room temperature and then observed for lines of precipitation.

Labeling of Antiserum

Fractionation of Antiserum for Enterotoxin B (42,44). Antiserum prepared as previously described using 95% pure enterotoxin B was used for fluorescent labeling.

An equal volume of $(NH_4)_2SO_4$ (54 g/71 ml) was added to an equal volume (10 ml) of cold undiluted antienterotoxin serum. This was done using a buret in an ice chamber at 4 C. The serum was continually stirred while adding the $(NH_4)_2SO_4$ solution dropwise. After addition of the ammonium sulfate solution, the precipitated globulin was placed in the refrigerator and allowed to aggregate 4 hr and then centrifuged at 1400 x g at 2 C for 1 min in an International Refrigerated centrifuge

model PR-2. Immediately after centrifugation, the supernatant fluid was removed and the precipitated globulin was dissolved in a volume of phosphate buffered saline (Appendix) equal to the original volume of serum. Cold ammonium sulfate of a volume equal to globulin was again added dropwise to the globulin. The globulin was allowed to aggregate overnight and then centrifuged in the refrigerated centrifuge at the same speed and time as previously described. The supernatant fluid was removed and the precipitated globulin was dissolved again in phosphate buffered saline using the smallest possible volume to prevent dilution of the globulin.

The ammonium sulfate was removed from the globulin by passing the globulin through a G-25 Sephadex column (31,53). The column was prepared by presoaking G-25 Sephadex gel (25 g of G-25) in distilled water overnight. The Sephadex was then poured into a 2 cm x 40 cm glass column with glass wool at bottom to form a column approximately 20 cm high, glass wool was then placed at top of the column.

Fractionated globulin (6 to 9 ml sample) was then added to the column and eluted with distilled water, phosphate buffered saline could not be used because it gave a precipitate with barium chloride. The column was kept in an ice jacket at 4 C at all times during elution. A void volume (liquid capacity of column) of approximately 40 to 45 ml was allowed to go through before collecting samples (usually the globulin could easily be seen by an increase in turbidity). Five milliliter samples were collected until 15 ml had been collected, then 2 ml samples were collected until the elute was positive for sulfate with

barium chloride (48). Usually the first 10 ml after the 40 ml void volume contained 95% of the globulin.

The Beckman DK-2A spectrophotometer was used to determine the approximate concentration of protein (OD of 1.4 = 1 mg/ml at 280 mu) in the eluate from the Sephadex column (32).

Labeling Globulin with Fluorescein Isothiocyanate (FITC) (12,34)

1. The protein concentration of fractionated serum was adjusted to a concentration of 10-30 mg/ml with saline.
2. To the chilled protein in an ice bath was added 0.5 M carbonate buffer in saline pH 9.5 (Appendix) to obtain 10% by volume.
3. FITC (BBL) powder was added to the buffered protein in the proportion of 12-25 ug FITC per milligram of protein.
4. The protein dye mixture was stirred with a magnetic stirrer for approximately 12 hr at 4 C.
5. The labeled globulin was then removed for further processing.

Removal of Unreacted Dye from Conjugate (43)

The unreacted dye was removed by gel-filtration through a Sephadex G-50 column. The Sephadex was prepared by suspending 10 g of gel in distilled water and washed free of "fines" by repeated sedimentation and decanting. The gel slurry was then poured into 2 cm x 40 cm column with glass wool at the bottom and allowed to settle (column height 17.5 cm), and then was equilibrated with phosphate buffered saline, pH 7.0 - 7.5. The column was placed in an ice jacket and cooled to 4 C. The conjugate was delivered to the top of the Sephadex and allowed to soak into the column. When the last portion of conjugate had soaked into the Sephadex elution was begun with cold (4 C) 0.0175 M

phosphate buffer at pH 6.3. A front of conjugated protein was seen to separate quickly from the unreacted dye, collection of conjugated protein was begun as soon as the colored fraction appeared in the elute and stopped as soon as the first colored band was eluted from the column (21).

Fractionation of Conjugated Globulin with DEAE Cellulose (56)

1. Dry DEAE cellulose (cellulose N,N-Diethylamino-ethyl ether, Eastman Organic Chemicals) approximately 1 g/20-30 mg of protein to be fractionated was sedimented for 30 min in distilled water and "fines" were decanted.

2. The cellulose was washed successively with 1.0 N NaOH, 1.0 N HCL, 1.0 N NaOH, and then brought to pH 7.0 with distilled water. Each washing was carried out with ten volumes of reagent for each initial volume of cellulose for 30 min with stirring. The cellulose was collected on a Buchner funnel using cheese cloth to retain cellulose after each wash.

3. The cellulose slurry was made up and stored in 0.0175 M phosphate buffer at pH 6.3.

4. The slurry was then poured into the column and allowed to pack. The column was placed in an ice jacket and allowed to cool.

5. The conjugated globulin was equilibrated against 0.0175 M phosphate buffer at pH 6.3. This was accomplished by eluting conjugated globulin from a Sephadex G-50 column.

6. The conjugate was placed on the cellulose column and eluted in three steps.

   Fraction I - Elution with 0.0175 M pH 6.3 phosphate buffer in distilled water

   Fraction II - Elution with 0.0175 M pH 6.3 phosphate buffer in 0.125 M NaCl

   Fraction III - Elution with 0.0175 M pH 6.3 phosphate buffer in 0.250 M NaCl

7. With each step a colored fraction was seen to emerge. When major portions of each colored fraction had been collected, the next higher salt concentration was added. The first two fractions were the only ones saved in this work.

8. Conjugated protein from the cellulose column is diluted and has to be concentrated. The conjugated globulin was placed in two and three quarter inch dialysis tubing and dialyzed against 30% polyethylene glycol 20,000 (Fisher Sci. Co., Fairlawn, N. J.) for 24 hr or until 20 ml remained.

9. The ratio of fluorescein to protein was determined at wavelengths of 280 mμ and 489 mμ using the Beckman DK-2A spectrophotometer.

10. Conjugated globulin was then stored in 1 ml aliquots at -20 C.

Note: Preparation of all buffers is described in the Appendix.

Staining of Organisms with Fluorescent Antibody (33,48)

1. Smears were made directly from broth cultures and food samples without pretreatment on Trident Fluoro-slides 1.0-1.2 mm in thickness. Each smear was uniformly spread using a wooden applicator within etched circles on the slide.

2. The slides were then placed in a 37 C incubator and allowed to dry for 1 to 2 hr. The slides were removed and fixed in 95% alcohol for 1 min.

3. After fixation with alcohol slides were allowed to dry and were then placed in petri dishes which had water soaked Whatman No. 1 filter paper in the covers.

4. The desired dilution of fluorescent antibody was then applied (one drop) to the slide and the petri dishes covered and incubated at room temperature for 30 min.

5. After 30 min, the excess antibody was allowed to run off the slide, by placing edge of slide against bibulous paper.

6. The slides were then placed in FA buffer (Difco) for 10 min with three changes of buffer.

7. Slides were then gently blotted dry using bibulous paper.

8. Immediately, one drop of FA mounting fluid (Difco) was placed in the center of the etched circle on the slide. A cover slip, Corning No. 1, 22 mm square was placed over the specimen.

9. Slides were then observed using an American Optical Fluorolume Illuminator (Model 645). Exciter filter used was a UGI (Corning #5840) with a Schott GG-9 barrier filter.

Membrane Filter Technique (29)

1. Food or broth supernatant (two drops) was mixed with one drop of FA (Fluorescent Antibody) on a cover slip and incubated at room temperature for 30 min in a petri dish (as for slides).

2. A large petri dish was filled with Whatman No. 1 filter paper (bottom) and was soaked with FA buffer. A millipore filter, 25 mm in diameter and with a pore size of 0.22 μ, was soaked in FA buffer then placed on filter paper in the petri dish. Two drops of FA buffer were added to the membrane filter to get complete contact with the filter paper.

3. The Ag-Ab mixture on the cover slip was then poured onto the center of the membrane filter avoiding spreading as much as possible. The membrane filter was then slowly washed with 20 drops of FA buffer.

4. After Ag-Ab mixture had disappeared an impression smear was made and observed as previously described.

Photographic Methods

All photographs were taken using a Kodak Colorsnap 35 Model 2 camera back with 5X adapter on an American Optical Series 10 microscope. Kodak high speed Ektachrome EH 135-36 (ASA 160) and TRI-X Pan TX 135-20 (ASA 400) films were used to photograph fluorescent organisms.

Good photographs were obtained using an exposure time of 1.5 to 3 min with TRI-X Pan film and an exposure time of 3 to 4 min with the Ektachrome film.

Culture Medium and Foods

Fisher brain heart infusion broth (BHI) (Fisher Scientific Co., Fairlawn, N. J.) and homogenized frozen shrimp (5%) were used throughout the investigation of nutritional and environmental effects on cell fluorescence and enterotoxin B production. Shrimp slurries were sterilized by autoclaving before use to eliminate all competitive growth. The argentometric method (silver nitrate titration) was used to titrate the sodium chloride concentration of a 5% shrimp slurry which was found to be 0.115%. All media were adjusted to a pH of 7.0 before inoculation.

A 5% homogenate of frozen peas and a beef pie were also investigated to determine the reliability of the FAT method.

General Procedures

Media Preparation. Cotton stoppered 250 ml erlenmyer flasks containing 100 ml of BHI broth and food slurries were used in all experiments. BHI broth was made up to 0.37% with distilled water and foods were homogenized for 2 min in distilled water (5% w/v) using an Oster blender. Before inoculation, all media were pretempered for 12 hr to the desired temperature.

Standard Inoculum. Both S. aureus strains 243 and S-6 were grown in 10 ml of BHI broth for 12 hr and 1 ml was diluted 1:100 with distilled water to facilitate washing of the cells. This dilution of organisms gave approximately 1×10^6 organisms/ml. In all experiments 1 ml of the dilution was added to each flask.

The Oakley-Fulthorpe double gel-diffusion technique was used to quantitate the amount of enterotoxin present in culture supernatant fluids of BHI broth and food slurries. Standard solutions containing 1 to 100 µg/ml of pure enterotoxin B were used as controls with each set of assay tubes.

Nutritional and Environmental Conditions

Sodium chloride was added to BHI broth and shrimp slurries at concentrations from 0 to 10% (w/v) in 1% increments to determine its effect on cell fluorescence and enterotoxin production. The concentrations of reagent grade NaCl described in both media were in addition to any amount already present in the medium (BHI 0.5%, shrimp 0.115%). Media were autoclaved after addition of NaCl.

The effect of aeration at 37 C was determined by comparing the enterotoxin concentrations and cell fluorescence of a shaken culture and a static culture. Shaken cultures were grown on a shaker water bath (150 rev/min). Static cultures were grown in a constant temperature water bath.

Glucose was added to 0.4% heart infusion broth (Difco) at concentrations from 0.1 to 0.6% in 0.1% increments to determine its effect on cell fluorescence and enterotoxin production. With this experiment the pH was recorded at 2 hr intervals so that pH effect could be distinguished from the glucose effect.

With sodium chloride, glucose, and aeration experiments, 2 ml aliquots were removed from the cultures every 2 hr after the first 4 hr of growth and cells were stained and observed for fluorescence and

the supernatants were assayed for enterotoxin.

The effect of carbon dioxide and nitrogen on growth and enterotoxin production in BHI broth was determined. A special flask system was used so that a positive pressure could be maintained in the flasks. The flask used was a screw cap side arm flask with cleaning port (Bellco No. 300). A rubber stopper used for serum bottles was inserted in the cleaning port. This allowed the evacuation of the flask using a syringe needle and the removal of aliquots with a syringe without disturbing the added atmosphere. Carbon dioxide and nitrogen were added to make up 25, 50 and 100% of the flask volume. Every 2 hr growth was monitered by making absorbance readings at 620 mμ and aliquots of 2 ml were removed and assayed for enterotoxin.

The effect of initial pH of BHI broth on cell fluorescence and enterotoxin production was determined by adjusting the pH from 5.9 to 9.0. The pH was adjusted with the following concentrations of $NaH_2PO_4 \cdot H_2O$/25 ml BHI* (28).

pH	g of NaH_2PO_4
5.1	1.3452
5.5	0.5758
6.0	0.2198
6.5	0.0693
6.9	0.0215
7-9	1N NaOH

* BHI was brought to 2% NaCl before pH adjustment

Aliquots of 2 ml were taken at 2 hr intervals and assayed for toxin, observed for fluorescence, and pH determinations were made.

Shrimp slurries and BHI broth cultures were incubated at 12 C, 25 C, 37 C, and 44 C for 6 days to determine the effect on cell fluorescence and enterotoxin production. Aliquots of culture were removed every day and every 2 hr as the cultures at the various temperatures began the early log phase of growth. With the higher temperatures, aliquots were removed at 2 hr intervals after the first 4 hr of growth.

Enterotoxin production and cell fluorescence in relation to time were determined in shaken cultures at 37 C with aliquots of culture being removed at 2 hr intervals for 24 hr. Smears were observed for cell fluorescence and aliquots were assayed for enterotoxin.

Five percent slurries of frozen peas and beef pie were used to determine if any interference would be obtained using other foods. Cultures were grown on the shaker water bath and cells observed for fluorescence after staining and the culture supernatants fluid assayed for enterotoxin.

specificity of labeled antiserum.

After cells were stained and observed they were given values of fluorescence based on visual observation. These values of fluorescence 0 to +4 were based on the brightness of cell fluorescence and fluorescent precipitates of Ag-Ab complexes. Representative fields for these fluorescent values are shown in Figures 1 to 4. These pictures were taken of the same culture at different periods of growth at the same exposure. Fluorescent precipitates which were also used in giving values of fluorescence to a field of cells are shown in Figures 5 and 6. These precipitates of labeled antibody-enterotoxin complexes became more prevalent with increasing cell age. The culture supernatant fluids were examined using the membrane filter technique and observed for fluorescent precipitates as shown in Figure 7. The number of these precipitates in a field increased as enterotoxin concentration increased.

The effect of cell age on the fluorescence of cells was not established until varying nutritional and environmental conditions were investigated. Under these conditions it was found that cells had to be observed early in their growth phase or no correlation could be established with enterotoxin production. Figures 1 to 4 demonstrate the increase in fluorescence with cell age from +1 to +4 through 4, 6, and 10 hr at 37 C in BHI broth.

Fig. 1. Staphylococcus aureus strain 243 cells from 4 hr BHI broth culture were stained with conjugate and given a +1 value, 1000X.

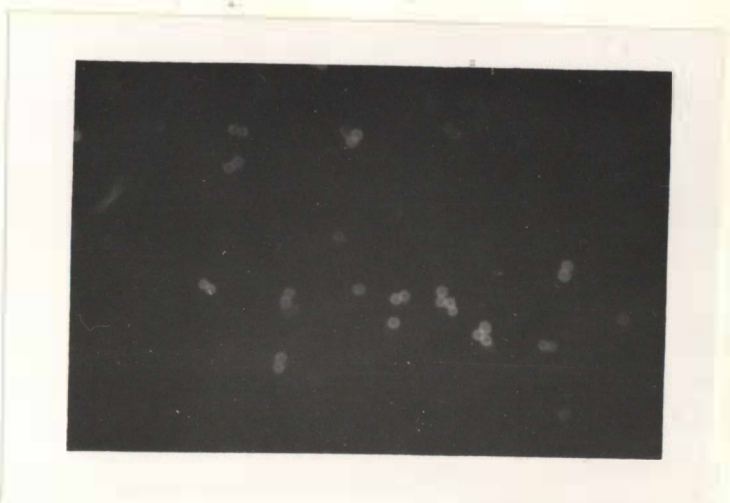

Fig. 2. Staphylococcus aureus strain 243 cells from 6 hr BHI broth culture were stained with conjugate and given a +2 value, 1000X.

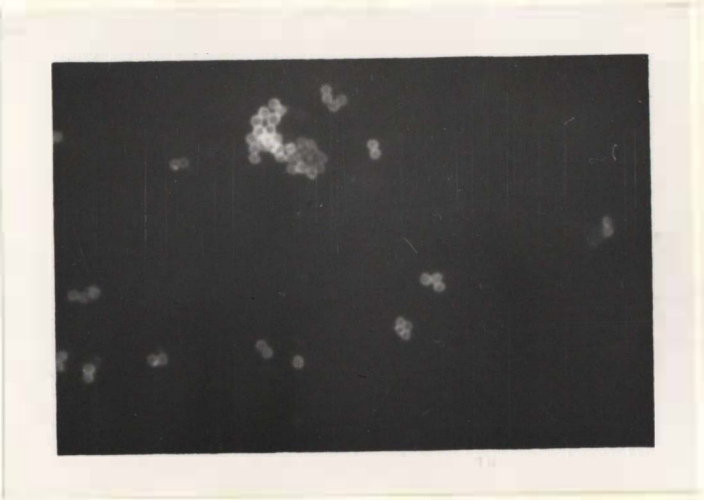

Fig. 3. Staphylococcus aureus strain 243 cells from 8 hr BHI broth culture were stained with conjugate and given a +3 value, 1000X.

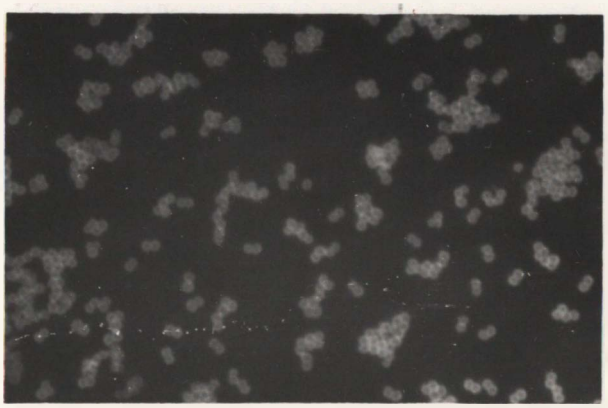

Fig. 4. Staphylococcus aureus strain 243 cells from 10 hr BHI broth culture were stained with conjugate and given a +4 value, 1000X.

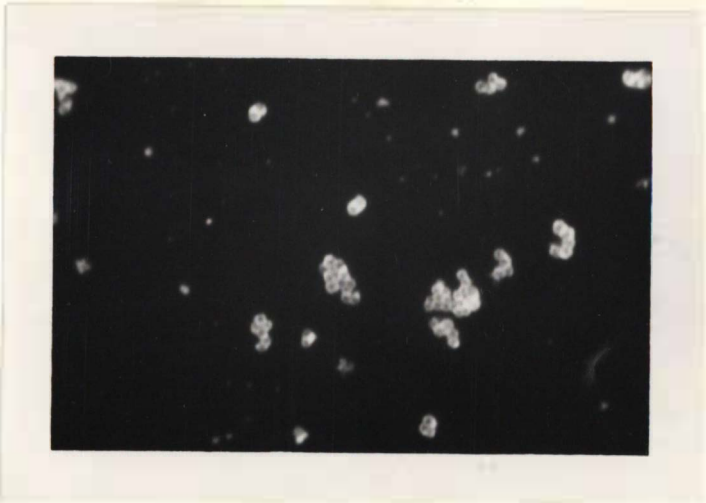

Fig. 5. Strong fluorescent area in group of strain S-6 cells from BHI broth, 1000X.

Fig. 6. Fluorescent precipitates around cells of strain S-6 grown in 5% shrimp slurry, 1000X.

Fig. 7. Fluorescent precipitates obtained from shrimp slurry supernatant of strain 243 using the membrane filter technique.

Effects of Nutritional and Environmental Conditions

Effect of NaCl on Cell Fluorescence and Enterotoxin B Production in BHI Broth and Shrimp Slurry. Sodium chloride concentrations from 0 through 10% had little effect on total growth of cells, although at NaCl concentrations greater than 3%, both cell fluorescence and enterotoxin production decreased rapidly with both strains and in both media (Fig. 8). During the first 12 hr of growth, cell fluorescence and enterotoxin production were directly proportional for both strains of *S. aureus* (Fig. 9, 10). However, after 16 hr of growth cells of strain S-6 began to show an increase in fluorescence which did not correlate with toxin production (Fig. 10). The fluorescent cells of strain S-6 at concentrations above 5% of NaCl may indicate that enterotoxin was being produced in undetectable amounts for gel-diffusion. Enterotoxin was formed in detectable amounts up to 7% NaCl with strain S-6 and up to

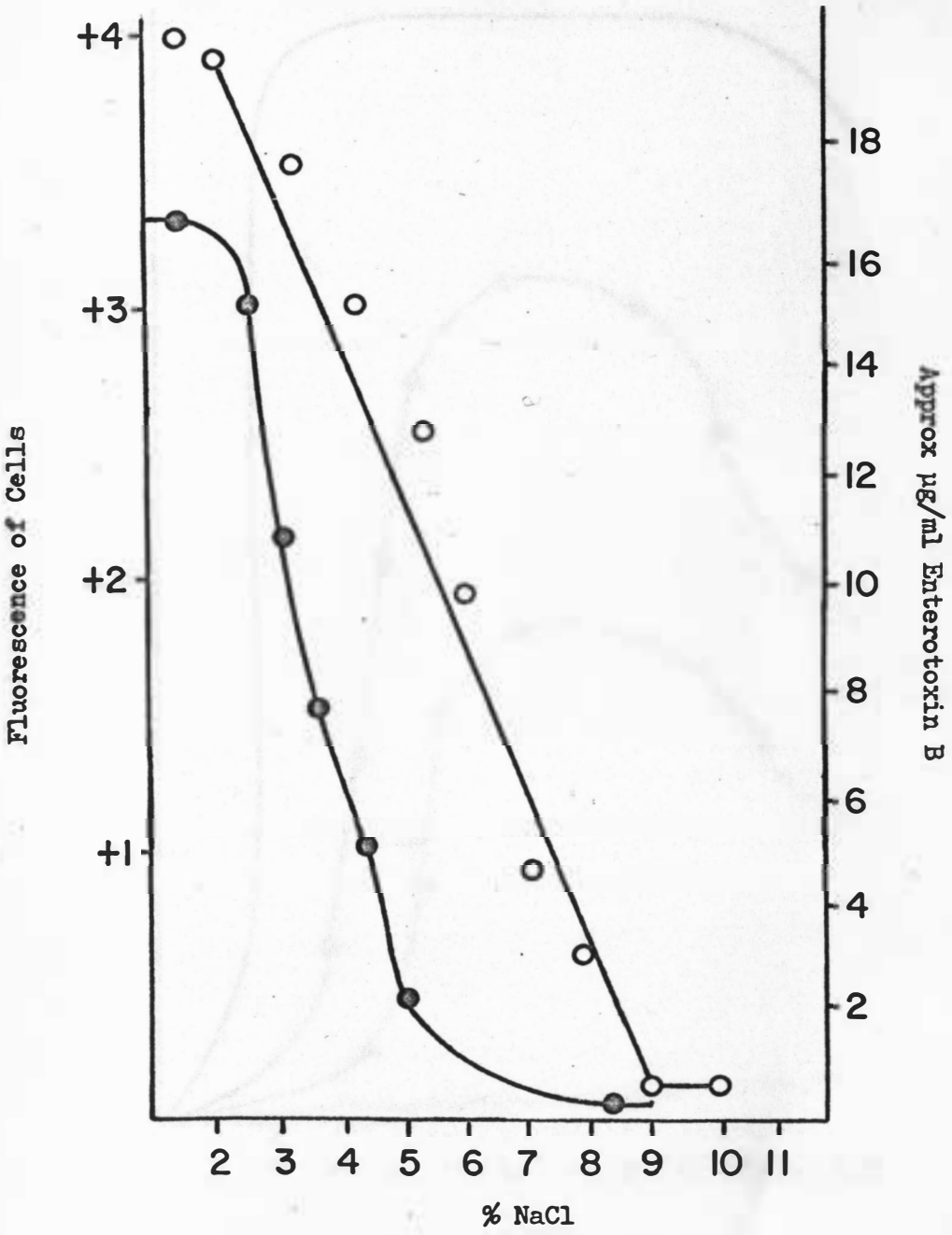

Fig. 8. Sodium chloride inhibition of cell fluorescence and enterotoxin B production with Staphylococcus aureus strains 243 and S-6 in BHI broth and shrimp slurries. Symbols: O , cell fluorescence at 10 hr; ● , enterotoxin B production at 24 hr.

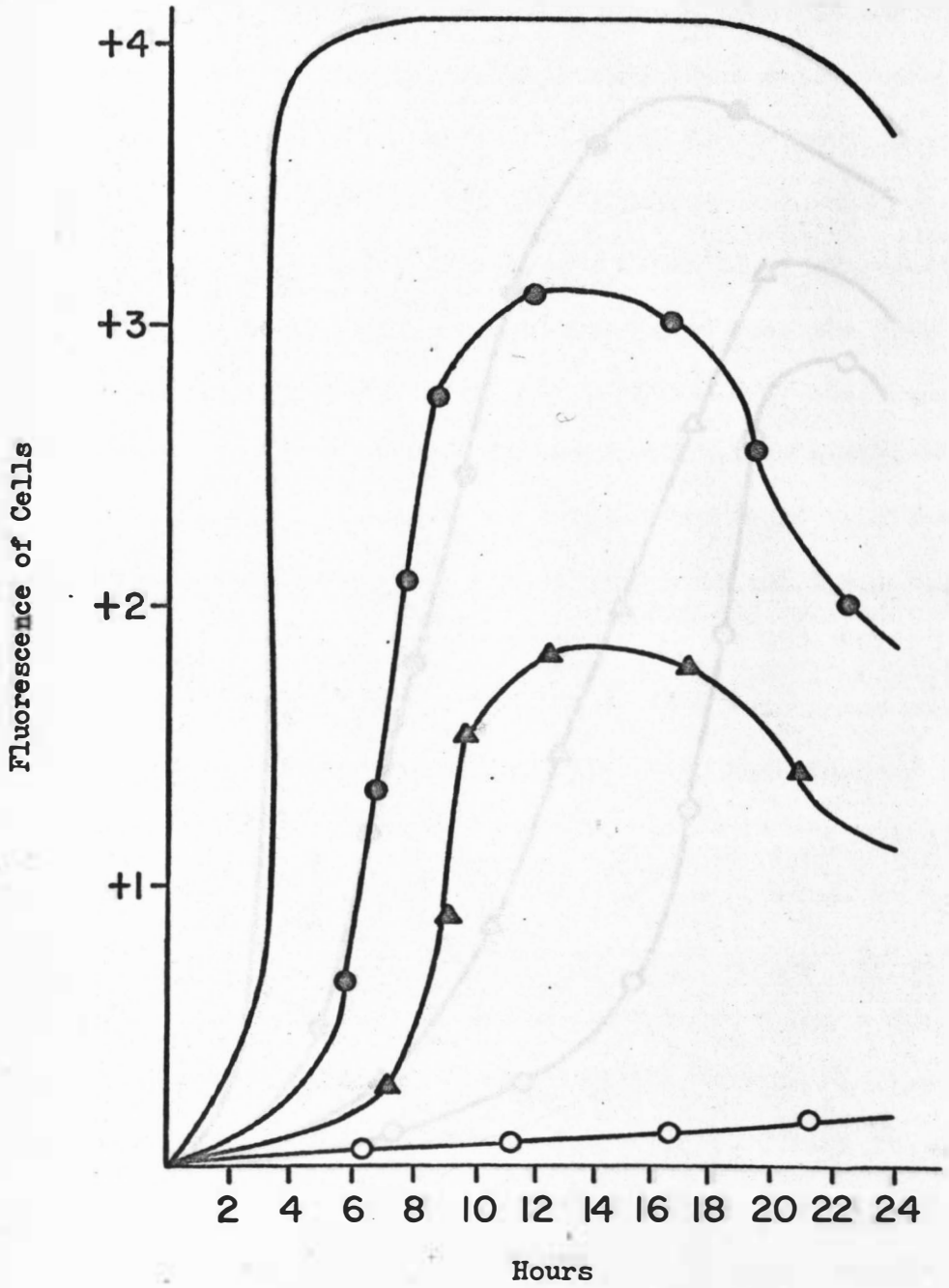

Fig. 9. Effect of increasing NaCl concentrations on cell fluorescence of strain 243 in shrimp slurries and BHI broth. Symbols: —, 0% NaCl; ●, 4% NaCl; ▲, 6% NaCl; ○, 10% NaCl.

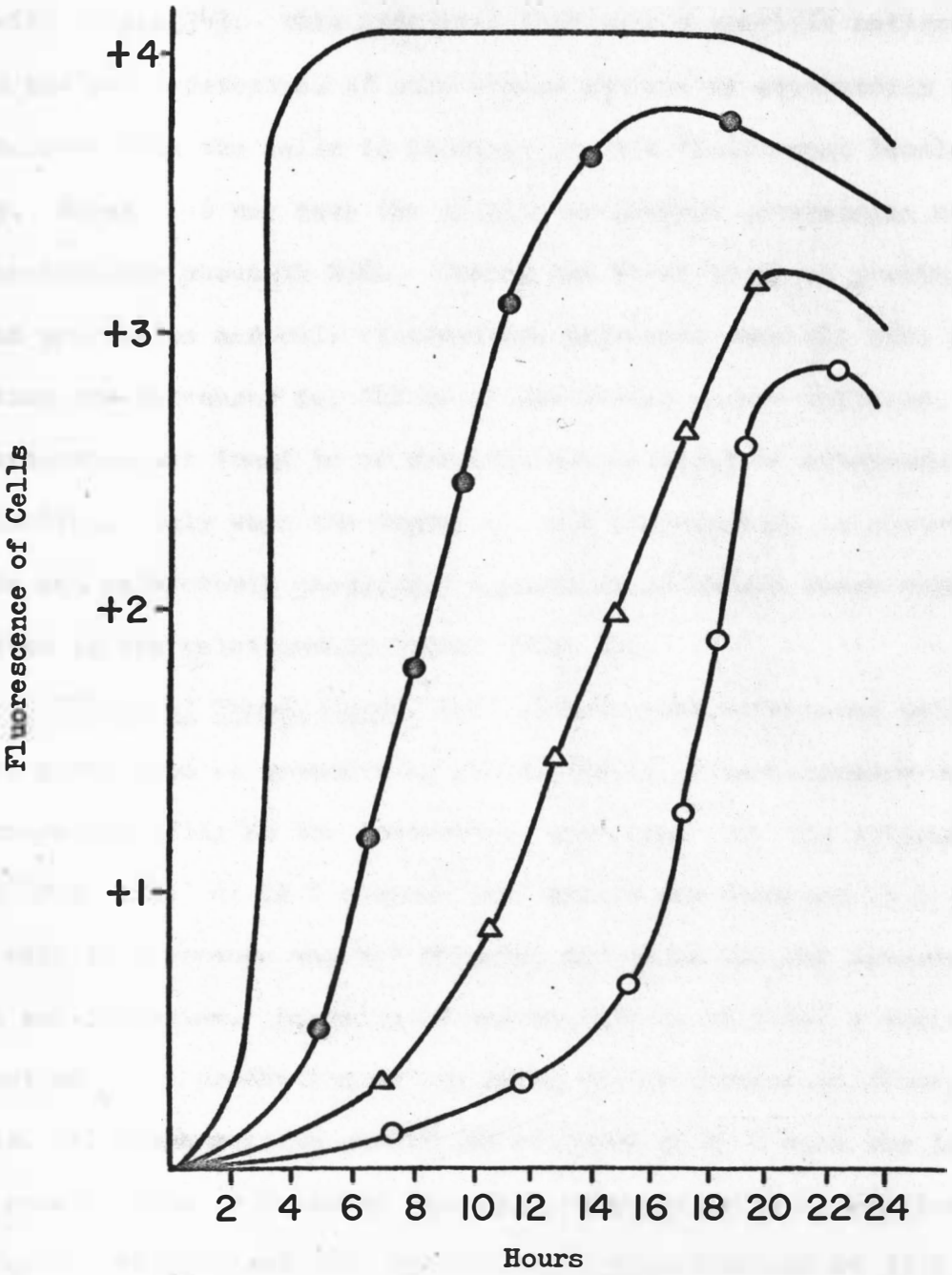

Fig. 10. Effect of increasing NaCl concentrations on cell fluorescence of strain S-6 in shrimp slurries and BHI broth. Symbols: — , 0% NaCl; ● , 4% NaCl; Δ , 7% NaCl; o , 10% NaCl.

6% with strain 243. This indicates that when a specific antiserum is used the rapid detection of very minute amounts of enterotoxin directly associated with the cells is possible using a fluorescent labeled antibody. Strain S-6 may have the ability to produce enterotoxin at concentrations above 7% NaCl. During the first 12 hr of growth, both toxin production and cell fluorescence decreased when the NaCl concentration was increased for BHI broth and shrimp slurry cultures. Cell fluorescence was found to be directly proportional to enterotoxin production. Only when the degree of cell fluorescence is observed at 10 hr and enterotoxin production assayed at 24 hr and these values plotted is the relationship linear (Fig. 8).

Effect of Temperature. Cell fluorescence correlated well with toxin production as measured by gel-diffusion at each temperature; both increased steadily as the temperature increased with the optimum at 37 C (Fig. 11). At 12 C maximum cell growth was obtained in 6 days but cell fluorescence was not observed and toxin was not detectable with gel-diffusion. Lowering of temperature to 12 C has a decisive effect on toxin production as was shown by the absence of fluorescent cells. Although maximum growth was obtained at 25 C with the logarithmic growth phase 12 hr later than that obtained at 37 C, the total production at 25 C was only one fourth of that obtained at 37 C (Table 1). At 25 C fluorescence reached a maximum value of +2 showing a good correlation between cell fluorescence and toxin production in BHI broth and shrimp slurries. At temperatures above 25 C, maximum toxin production and cell fluorescence were reached within 24 hr.

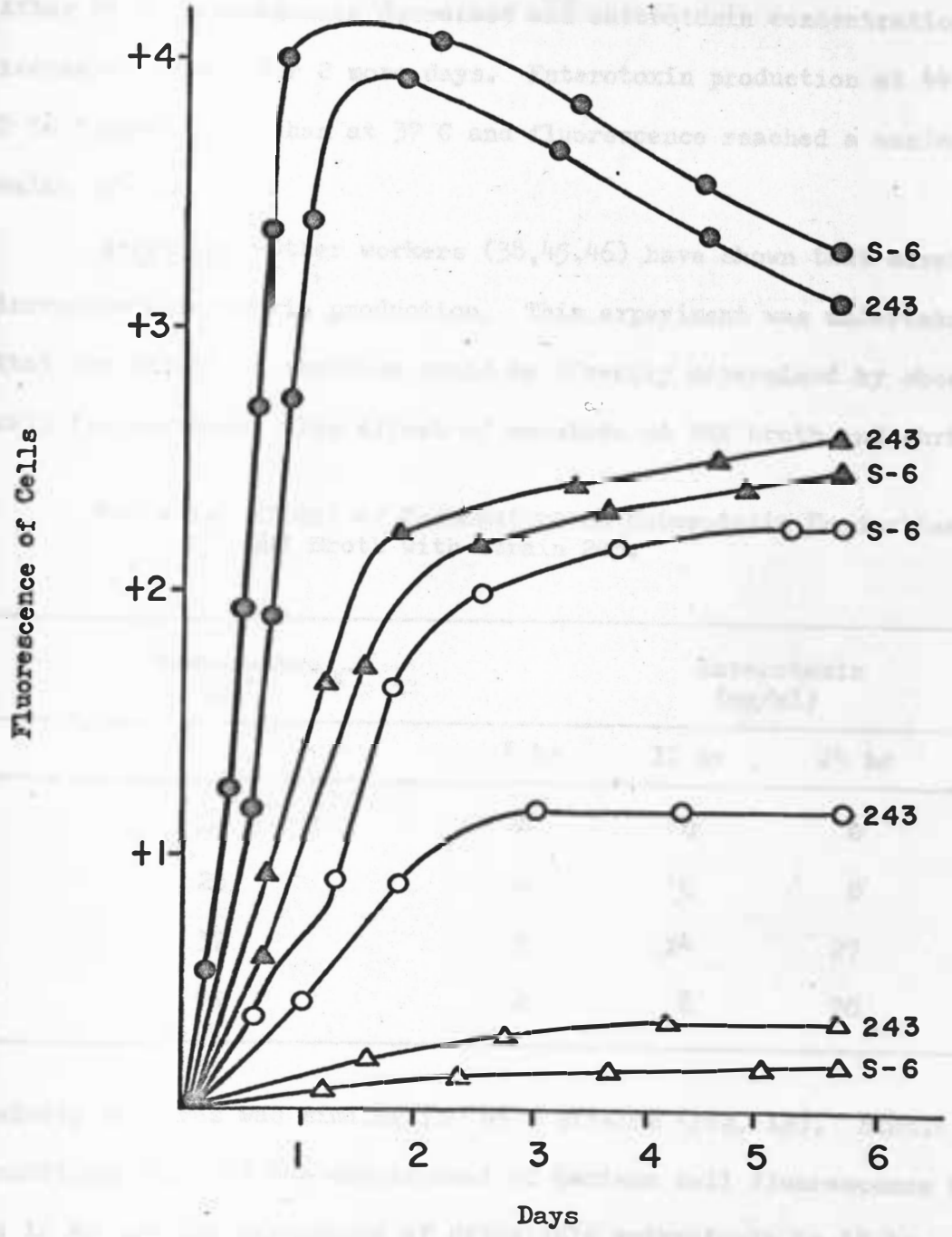

Fig. 11. Effect of incubation temperature on cell fluorescence with strains 243 and S-6 in BHI broth and shrimp slurries. Symbols: Δ , 12 C; O , 25 C; ● , 37 C; ▲ , 44 C.

After 24 hr fluorescence decreased and enterotoxin concentration increased slowly for 2 more days. Enterotoxin production at 44 C was 2 to 5 µg/ml less than at 37 C and fluorescence reached a maximum value of +3.

Aeration. Other workers (38,45,46) have shown that aeration increases enterotoxin production. This experiment was undertaken so that the effect of aeration could be directly determined by observing cell fluorescence. The effect of aeration on BHI broth and shrimp

Table 1. Effect of Temperature on Enterotoxin Production in BHI Broth with Strain 243.

Temperature (C)	Enterotoxin (µg/ml)			
	8 hr	12 hr	24 hr	48 hr
12	0	0	0	0
25	0	0	0	9
37	5	14	27	36
44	2	8	20	28

slurry cultures was similar for both strains (Fig. 12). Static growth conditions delayed the development of maximum cell fluorescence by 10 to 12 hr and the appearance of detectable enterotoxin by 18 hr. Under static growth conditions enterotoxin was first detectable at 24 hr, whereas, in aerated cultures toxin was detectable within 6 hr. Development of maximum cell fluorescence in shrimp slurry cultures was reached at a time later than with BHI broth cultures.

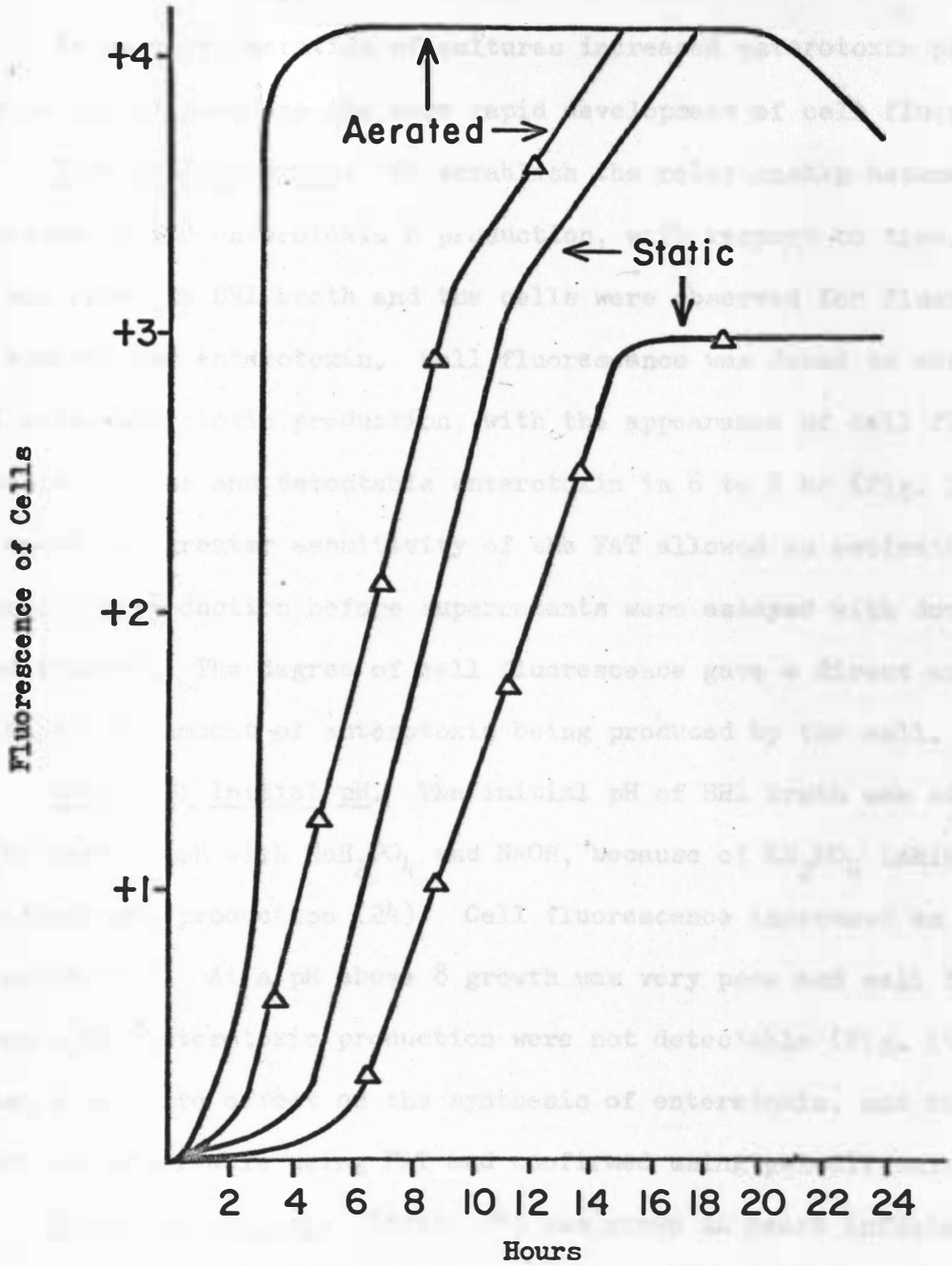

Fig. 12. Effect of aeration and static conditions on cell fluorescence with strains 243 and S-6 at 37 C. Symbols: —— , BHI broth; Δ , 5% shrimp slurries.

In summary, aeration of cultures increased enterotoxin production and allowed for the more rapid development of cell fluorescence.

Time of Incubation. To establish the relationship between cell fluorescence and enterotoxin B production, with respect to time, strain 243 was grown in BHI broth and the cells were observed for fluorescence and assayed for enterotoxin. Cell fluorescence was found to correlate well with enterotoxin production, with the appearance of cell fluorescence in 4 hr and detectable enterotoxin in 6 to 8 hr (Fig. 13). The speed and greater sensitivity of the FAT allowed an estimation of enterotoxin production before supernatants were assayed with double gel-diffusion. The degree of cell fluorescence gave a direct correlation to the amount of enterotoxin being produced by the cell.

Effect of Initial pH. The initial pH of BHI broth was adjusted to the desired pH with NaH_2PO_4 and NaOH, because of KH_2PO_4 inhibition of enterotoxin production (24). Cell fluorescence increased as pH increased to 8. At a pH above 8 growth was very poor and cell fluorescence and enterotoxin production were not detectable (Fig. 14). The pH has a definite effect on the synthesis of enterotoxin, and this effect was observable using FAT and confirmed using gel-diffusion.

Effect of Glucose. Strain 243 was grown in heart infusion broth with added glucose (0.1 to 0.6%) for 24 hr. Glucose can have two effects on enterotoxin production: it can provide the cell with a carbon source rather than using amino acids for carbon, or a pH effect from the acid that was produced. The effects of glucose on cell fluorescence and enterotoxin production are shown in Table 2. As

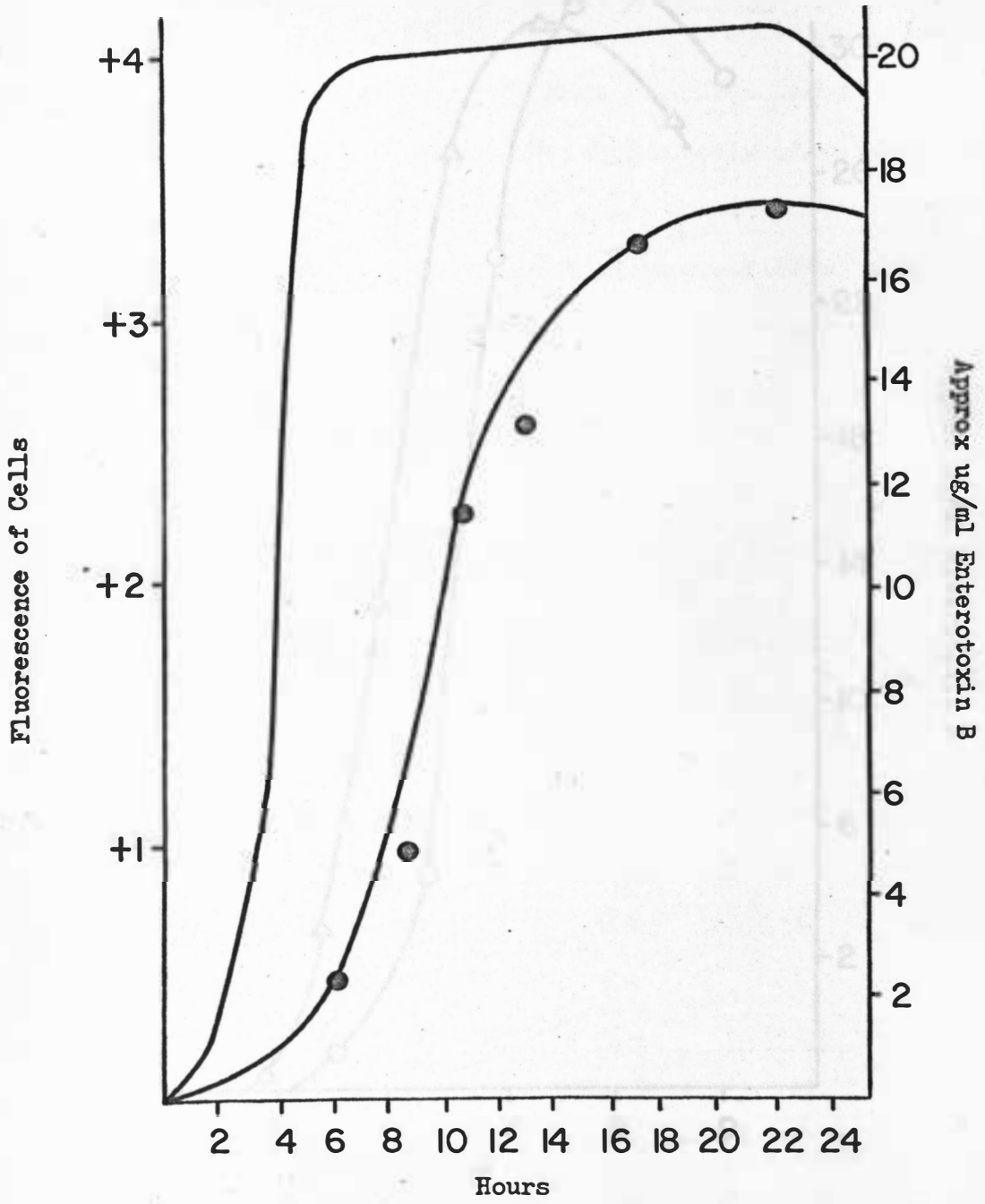

Fig. 13. Cell fluorescence and enterotoxin B production versus time with strain 243 in BHI broth at 37 C. Symbols: —, cell fluorescence; ● , enterotoxin B.

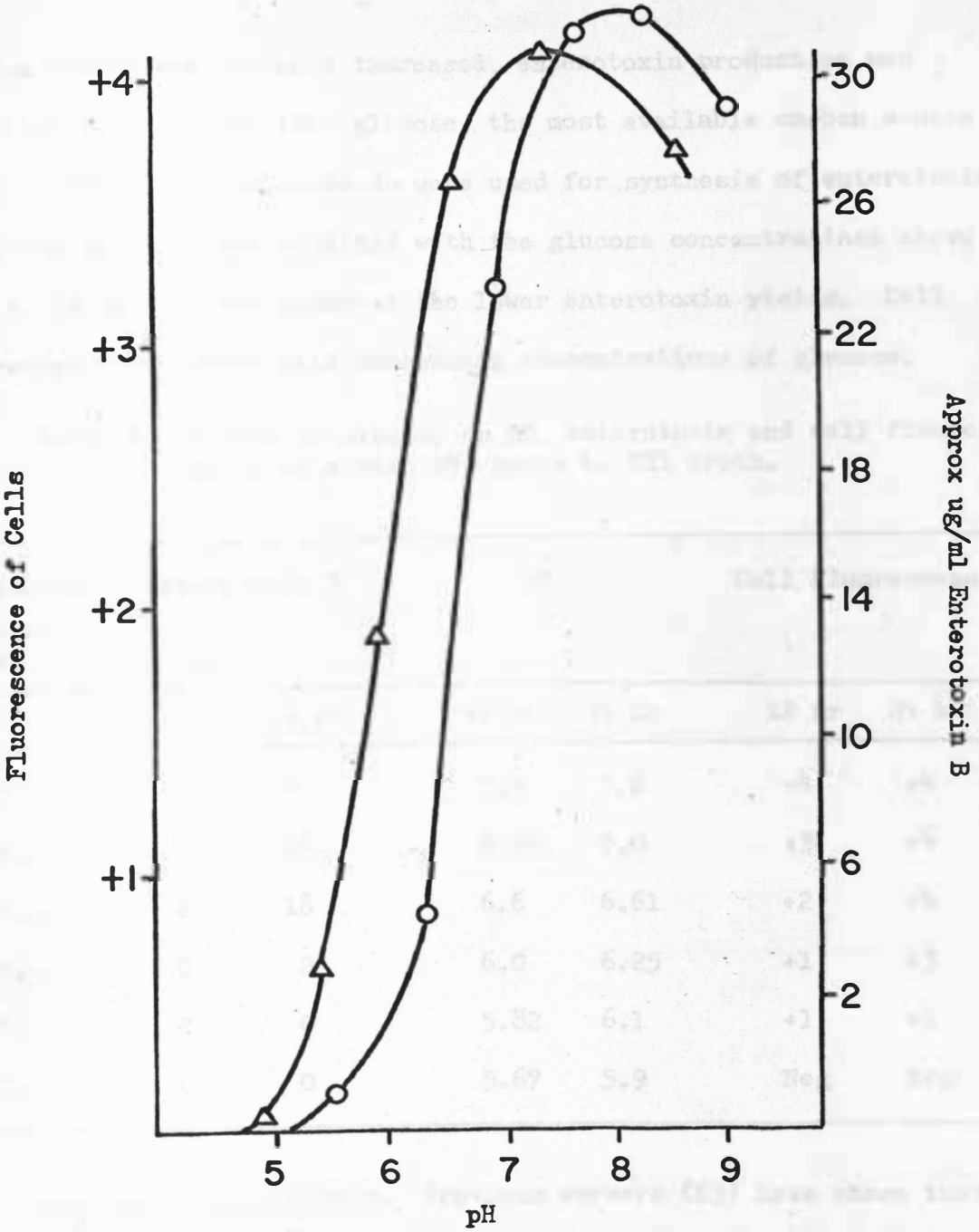

Fig. 14. Effect of initial pH on enterotoxin B production and cell fluorescence of strains 243 and S-6 in BHI broth at 37 C. Symbols: Δ , cell fluorescence at 10 hr; O , enterotoxin B at 24 hr.

glucose concentrations were increased, enterotoxin production was inhibited. Indicating that glucose, the most available carbon source was used before the amino acids were used for synthesis of enterotoxin. The lower pH which was obtained with the glucose concentrations above 0.3% could also be the cause of the lower enterotoxin yields. Cell fluorescence decreased with increasing concentrations of glucose.

Table 2. Effect of glucose on pH, enterotoxin and cell fluorescence of strain 243 grown in BHI broth.

Glucose conc (%)	Enterotoxin B (µg/ml)		pH		Cell Fluorescence	
	12 hr	24 hr	12 hr	24 hr	12 hr	24 hr
0.1	12	30	7.5	7.6	+4	+4
0.2	5	26	6.95	7.0	+3	+4
0.3	2	18	6.6	6.61	+2	+4
0.4	0	2	6.0	6.25	+1	+3
0.5	0	0	5.82	6.1	+1	+1
0.6	0	0	5.67	5.9	Neg	Neg

CO_2 and N_2 Atmospheres. Previous workers (63) have shown that production of enterotoxin by S. aureus in vacuum packaged foods is inhibited. A comparison of CO_2 and N_2 atmospheres versus atmosphere was made to determine if it is the N_2 or CO_2 remaining in a vacuum packed food which inhibits enterotoxin production. The two strains were grown in BHI broth under the two atmospheres and the results

obtained are shown in Tables 3 and 4. The data given in the tables show that fluorescence and enterotoxin have no relationship when cells are grown under the two atmospheres. Carbon dioxide may allow the synthesis of enterotoxin and its immediate release from the cell, whereas nitrogen may inhibit the release of enterotoxin from the cell, but the cell may have the capability to produce enterotoxin. At concentrations above 25% both gases decreased enterotoxin production with N_2 causing complete inhibition. The effect of these two gases on enterotoxin production is being subject to further investigation.

Table 3. Effect of CO_2 on enterotoxin production and cell fluorescence of strains 243 and S-6 in BHI broth.

CO_2 conc (% added)	Enterotoxin B (ug/ml)			Cell Fluorescence		
	8 hr	12 hr	24 hr	8 hr	12 hr	24 hr
Control	5	17	30	+2	+4	+4
25	4	15	26	+1	+1	+1
50	2	12	20	Neg	+1	+1
100	0	9	14	Neg	Neg	Neg

Table 4. Effect of N_2 on enterotoxin production and cell fluorescence of strains 243 and S-6 in BHI broth.

N_2 conc (% added)	Enterotoxin B (ug/ml)			Cell Fluorescence		
	8 hr	12 hr	24 hr	8 hr	12 hr	24 hr
Control	5	17	30	+2	+4	+4
25	3	12	24	+2	+3	+4
50	0	0	0	+1	+3	+3
100	0	0	0	+2	+3	+3

Beef Pie and Frozen Pea Slurries. These two foods were examined to determine if the FAT can be applied to detect cells and enterotoxin in the presence of possible interfering food proteins. The cell fluorescence for both strains of S. aureus cells was distinct from the food particles with both beef pie and pea slurries. The cell fluorescence increased with time for BHI and for shrimp.

Enterotoxin was not detectable in the three food cultures when assayed by double gel-diffusion. This would indicate the need for extraction and concentration of the enterotoxin. Enterotoxin was detectable in the food slurry cultures using the membrane filter technique; the number of fluorescent precipitates were found to correlate with degree of cell fluorescence. Observation of food slurry smears revealed that cells were associated with food particles indicating the enterotoxin is probably adsorbed to the food.

Extraction from Food. Enterotoxin B was extracted and concentrated from the shrimp using the method of Casman (11). The extraction of enterotoxin was performed to verify that the 5% shrimp slurries would support the production of enterotoxin and to verify that results obtained using the FAT were valid. Strain 243 grown in a 5% shrimp slurry for 24 hr was used for the extraction procedure. After the extraction had been completed the eluate was concentrated and assayed for enterotoxin using double gel-diffusion. The enterotoxin concentration in the 5% shrimp culture was found to be approximately 2 ug/ml. Although extraction procedures were not run on all the shrimp cultures of the study, the results obtained with fluorescent staining seem to agree with actual concentrations of enterotoxin.

The results obtained using the FAT to directly determine enterotoxin production by observing cell fluorescence indicates that there is a direct correlation between fluorescence and enterotoxin being produced by the cells.

Further studies for the effect of N_2 and CO_2 will be needed to determine the actual mechanism involved. If this mechanism can be determined, it may be possible to develop techniques of processing food and preventing enterotoxin production.

A FAT study of *S. aureus* cells grown in many foods and in competition (35) with the natural flora of organisms is especially recommended.

Another application which should be investigated is the possibility of using rhodamine-labeled antibody against enterotoxin A. This would allow the observation of a mixed culture and the determination if both types of enterotoxin producing cells are present. Rhodamine is a red fluorescent dye and fluorescein isothiocyanate is a green fluorescent dye. This would allow for a distinct contrast of cells in a mixed population.

CONCLUSIONS

1. A fluorescein isothiocyanate labeled antibody was prepared and found to be highly specific for *S. aureus* cells producing enterotoxin B.
2. Concentrations of NaCl 0 to 10% were found to cause a proportional decrease in cell fluorescence and enterotoxin B production. A linear relationship was found when fluorescence was observed at 10 hr and enterotoxin was assayed at 24 hr.
3. Maximum cell fluorescence and the highest rate of enterotoxin B production were reached at 37 C in the shortest period of time.
4. Aeration enhanced the rate of development for both cell fluorescence and enterotoxin B production.
5. A positive correlation was found between cell fluorescence and enterotoxin B production during the first 12 hr of growth in BHI broth at 37 C.
6. As the initial pH was increased from pH 5.5 to pH 8 cell fluorescence and enterotoxin B production increased with a maximum for both at a pH of 7.5.
7. Increasing glucose concentrations were accompanied by a decrease in both cell fluorescence and enterotoxin B production.
8. *S. aureus* strains 243 and S-6 grown under atmospheres of over 50% N_2 and CO_2 gave results different than those obtained with air. Under an atmosphere of N_2 cells were found to fluoresce but produced no detectable enterotoxin B. A CO_2 atmosphere allowed enterotoxin B production but inhibited cell fluorescence.

9. The fluorescent antibody technique was found applicable and reliable in rapidly detecting enterotoxin B in food and culture medium and was more rapid than the double gel-diffusion method.

10. Enterotoxin B in shrimp slurries was not detectable with double gel-diffusion until it was extracted and concentrated using carboxymethyl cellulose and the method of Casman.

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APPENDIX

Buffers and Solution for Fluorescent Antibody Preparation

Phosphate - buffered saline, pH 7.5, 0.01 M buffer

Na_2HPO_4 (anhydrous; MW, 141.96)	1.2 g
$NaH_2PO_4 \cdot H_2O$ (MW, 137.99)	0.22 g
NaCl (MW, 58.44)	8.50 g
Distilled H_2O to make	1000 ml

Carbonate buffer, pH 9.5, 0.5 M

a) Dissolve 5.3 g of Na_2CO_3 (anhydrous; MW, 105.99) in 100 ml of 0.85% NaCl
b) Dissolve 4.2 g of $NaHCO_3$ (anhydrous; MW, 84.01) in 100 ml of 0.85% NaCl
c) Add 5.8 ml of (a) to 10 ml of (b) and adjust to pH 9.5

Phosphate buffer, pH 6.3, 0.0175 M (for DEAE chromotography)

a) Dissolve 2.48 g of Na_2HPO_4 in 1 liter of water
b) Dissolve 2.41 g of NaH_2PO_4 in 1 liter of water
c) Add 1 part of (a) to 2 parts of (b) and adjust to pH 6.3

Saline solutions (for DEAE chromotography)

a) Prepare 2 M stock solution by dissolving 11.69 g of NaCl in 100 ml of phosphate buffer, pH 6.3
b) To prepare 0.125 M NaCl mix 6.25 ml of 2 M NaCl with 93.75 ml of phosphate buffer, pH 6.3
c) To prepare 0.250 M NaCl mix 12.5 ml of 2 M NaCl with 87.5 ml of phosphate buffer, pH 6.3